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Targeted Magnetic Hyperthermia for Lung Cancer

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14. ABSTRACT
The hypothesis of this research is that inhalable SPIO nanoparticles surface-functionalized with EGFR targeting ligand, when exposed to an appropriate magnetic field, will enable highly efficient and specific tumor cell death by hyperthermia. In the second year of this three year research program, we have demonstrated effective cellular uptake and tumor cell kill with EGFR-targeted SPIO particle-mediated magnetic hyperthermia. Additionally, we have shown the effectiveness of EGFR targeting in enhancing the lung tumor concentration of SPIO particles in a mouse lung tumor model. Future studies will examine the in vivo efficacy of targeted SPIO particle-mediated hyperthermia in a lung tumor model.

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INTRODUCTION

Despite significant advances in diagnostic techniques and the discovery of new molecularly targeted therapies, lung cancer (specifically, non-small cell lung cancer; NSCLC) is a leading cause of cancer-related deaths in the United States. Poor response rates and survival with current treatments clearly indicate the urgent need to develop an effective means to treat NSCLC. Magnetic hyperthermia (MH) is a novel non-invasive approach for ablation of lung tumors, and is based on heat generation by magnetic materials, such as superparamagnetic iron oxide (SPIO) nano particles, when subjected to an alternating magnetic field. However, inadequate delivery of magnetic nanoparticles to tumor cells can result in sub-lethal temperature change and induce resistance. Additionally, non-targeted delivery of these particles to the healthy tissues can result in toxicity. To overcome these problems, we propose to use aerosol-based, tumor-targeted SPIO nanoparticles to induce highly selective tumor cell kill. Tumor cell specificity will be achieved by targeting SPIO nanoparticles to the epidermal growth factor receptor (EGFR), whose overexpression has been observed in as many as 70 % of NSCLC patients.

Hypothesis: *Inhalable SPIO nanoparticles surface-functionalized with EGFR targeting ligand, when exposed to an appropriate magnetic field, will enable highly efficient and specific tumor cell death by hyperthermia.*

Specific Aims: The specific aims of this research are: (1) to fabricate aerosol formulation of EGFR-targeted SPIO nanoparticles, (2) to characterize *in vivo* tumor specificity of inhaled EGFR-targeted SPIO nanoparticles, and (3) to determine the *in vivo* anticancer efficacy of inhaled EGFR-targeted SPIO nanoparticles.

BODY

Summary of tasks completed in year one

Water dispersible SPIO nanoparticles were synthesized by chemical precipitation of iron chlorides by a strong base and coated with myristic acid and Pluorinic ® to prevent oxidation of iron oxide and to impart aqueous dispersibility. They were characterized for size (transmission electron microscopy, dynamic light scattering), composition (iron assay, infrared spectroscopy), magnetic properties (vibrating sample magnetometry) and heating rates. The effect of MH on overall tumor cell kill was determined in A549 cells (NSCLCs) based on the amount of lactate dehydrogenase released by the dead cells in the medium and the 7-aminoactinomycin D positive fraction determined using flow cytometry. Mode of cell death was determined using the annexin-FITC (stains apoptotic cells) and propidium iodide (stains necrotic and advanced apoptotic cells) staining followed by flow cytometric analysis. Targeted and non-targeted SPIO nanoparticles were synthesized and characterized. Preliminary studies demonstrated the effectiveness of epidermal growth factor receptor (EGFR) targeting in the enhancing the tumor cell uptake of the SPIO nanoparticles. These preliminary studies were further confirmed in Year 2 of the project.

Year 2 Progress

We have made significant progress in completing the proposed studies. The results of these studies are presented below.

Specific Aim #1: Fabricate aerosol formulation of EGFR-targeted SPIO nanoparticles

Task 3: Demonstration of receptor-binding characteristics

Demonstration of the role of EGFR in tumor cell uptake of the functionalized nanoparticles

A549 cell were plated in a 6-well plate 4 hours before the start of the study. Cells were washed with phosphate buffered saline (PBS) to remove non-adherent cells and 1 mg (magnetite equivalent) of targeted SPIO particles (TPCP), scrambled peptide-conjugated particles (SPCP), particles without any peptide or TPCP + excess targeting peptide were added to the cells in a total volume of 2 ml of cell culture medium containing 5% FBS. The plates were incubated on ice for 30 minutes, washed three times with PBS and incubated at 37°C for an additional 45 minutes. At the end of incubation, the cells were lysed with 400 µl of RIPA buffer and assayed for the total cell protein content and the iron content[1] (by iron assay procedure discussed before) in the lysate. The iron oxide uptake by the cells was normalized to the total protein content. As can be seen from **Fig. 1**, cellular uptake of the EGFR targeted SPIO NP was 4.5-fold higher than the corresponding controls. The conjugation of scrambled peptide did not result in enhancement of particle uptake into cells, and the presence of excess targeting ligand decreased the cell uptake of TPCP, showing the specific role of EGFR in tumor cell uptake of targeted nanoparticles.

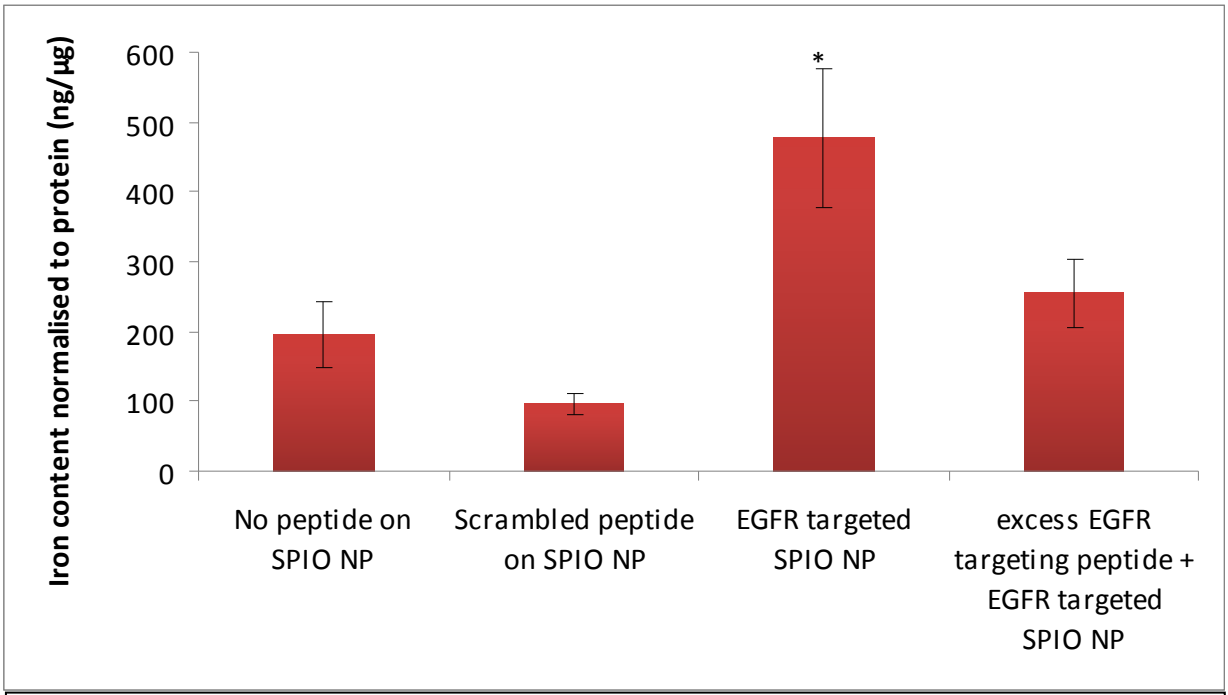

Fig 1: Effect of EGFR targeting ligand on SPION anoparticle (NP) cell uptake. n = 3 for each treatment group. Cellular uptake of EGFR targeted SPIO NP was significantly higher than that for other groups ($p < 0.01$).

To determine the benefit of EGFR targeting on the effectiveness of magnetic hyperthermia, plated A549 cells were incubated with TPCP and SPCP for 30 minutes at 4°C, washed three times with PBS, incubated at 37°C for 45 minutes and subjected to an alternating magnetic field (AMF) of 6 kA/m at 386 kHz frequency. Following AMF exposure, 10 μM propidium iodide was added to the cells and observed under the fluorescent microscope. The plates were incubated at 37°C overnight and imaged again after 24 hrs. As seen from **Fig. 2**, EGFR targeting lead to enhanced accumulation of SPIO NP (dark spots) on cells and higher propidium iodide uptake by the cells following exposure to magnetic field, indicating enhanced cell death.

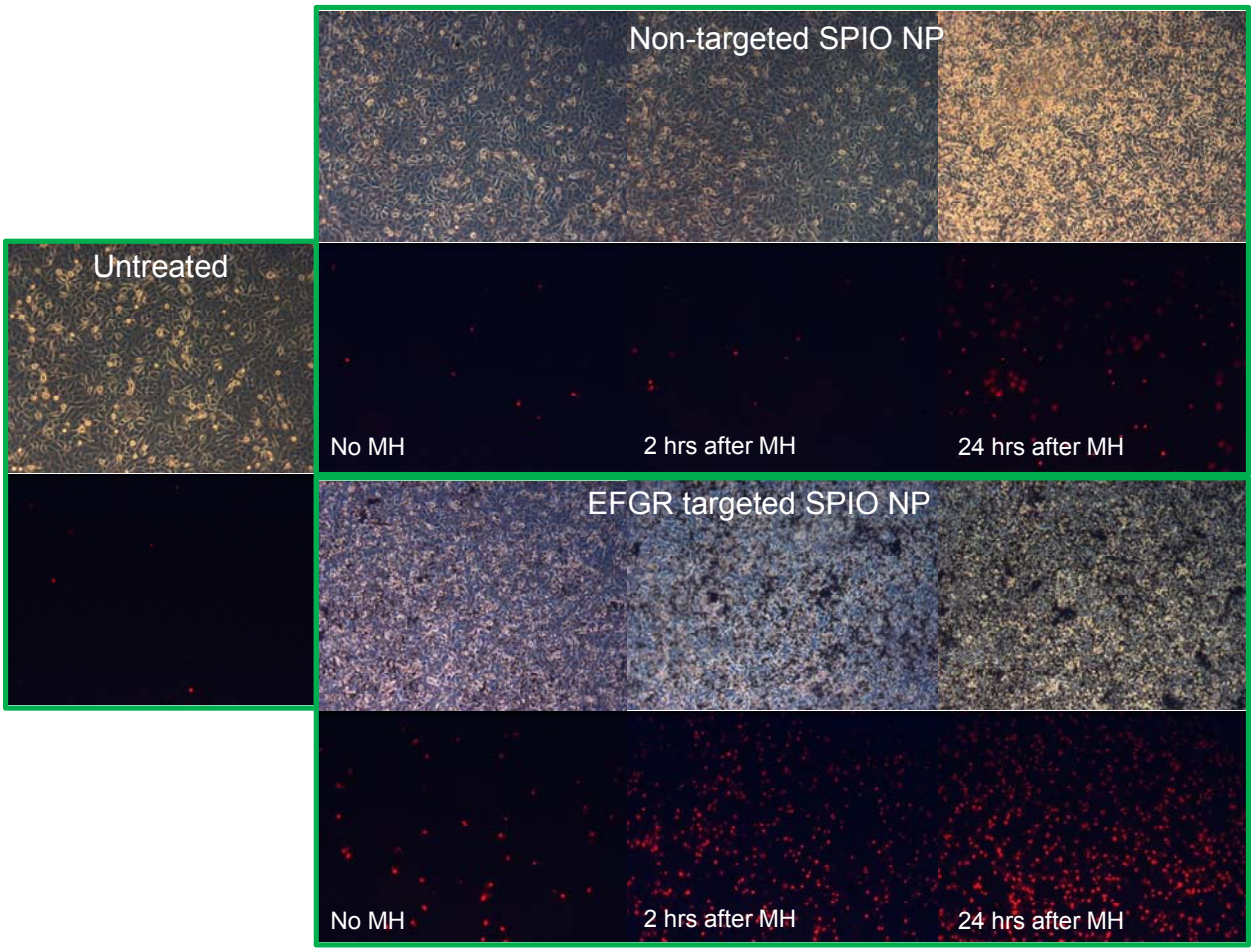

Fig 2: Microscopic images of all cells (bright field) and propidium iodide positive cells (red fluorescence) after magnetic hyperthermia using TPCP or SPCP. EGFR targeting results in enhanced adherence of particles on the cells and higher cell kill 2 hour and 24 hour after magnetic hyperthermia.

Specific Aim #2: Characterize *in vivo* tumor targeting of inhaled EGFR-targeted SPIO nanoparticles

Task 1: Study pharmacokinetics of targeted SPIO nanoparticles following inhalation delivery

Orthotopic lung tumor model

A mouse orthotopic lung tumor model was used in this study [2]. Lung tumor cells that have been stably transfected with fire-fly luciferase were used to induce tumors to facilitate the visualization of the tumor cells in live animals using bioluminescence imaging. *A549-luc-C8 Bioware® Cell Line (Caliper Lifesciences) is a luciferase expressing cell line derived from A549 human lung squamous carcinoma cells by stable transfection of the North American Firefly Luciferase gene expressed from the CMV promoter.* Intravenous injection of a 5 X 10^6 A549-luc-C8 cells led to detectable bioluminescence increase in the lungs by 2 weeks. The bioluminescence profile of the lung tumor is shown in **Fig. 3** as a function of time (in weeks) after tumor cell injection. The rate of tumor growth can be controlled by adjusting the number of cell injected.

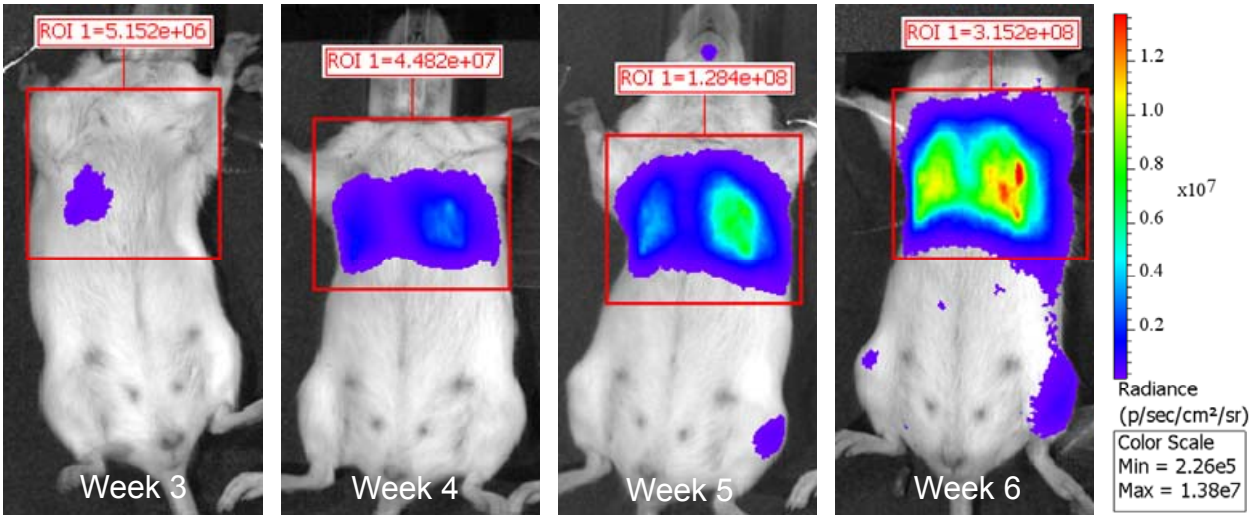

Fig 3. Bioluminescence profile of mouse lung following intravenous injection of A549-C8-Luc lung adenocarcinoma cells.

Routes of lung delivery of SPIO NP

Distribution and clearance of TPCP and SPCP were studied following two different routes of lung delivery – tracheal instillation[3] and aerosol[4].

In tracheal instillation, the mice were anesthetized and placed on a rodent intubation stand, supported by their incissors. The tongue was rolled out using a sterile cotton swab and held between the fingers. A fiber-optic light and stylus (BioLite, BioTex, Inc., TX) connected to an endotracheal tube was used to visualize the tracheal opening and to carefully insert the attached endotracheal tube into the trachea. An inflation bulb was used to confirm the proper insertion of the endotracheal tube by monitoring the inflation of the thoracic region on gently pushing in air through the tube. TPCP or SPCP (50 µl or 10 mg/ml iron oxide equivalent) were

instilled through the endotracheal tube. The mice were kept upright for a minute to prevent back-flow of the liquid and then they were placed on a heating pad to maintain their body temperature to ensure faster recovery from anesthesia.

In aerosol-mediated delivery of SPIO particles, an in-house assembly was designed for "nose-only" aerosol exposure in mice. Aerosol was generated by ultrasonic atomization[5]. A pyrex glass baffle was constructed in-house and placed in the water bath, directly over a 1.7 MHz ultrasonic transducer. About 13 ml of SPIO NP dispersion containing 8 mg magnetite per ml was loaded into the baffle. Compressed air directed into the baffle at a flow rate of 0.5 L/min (as measured by an inline flow meter) entrained the aerosol droplets containing the SPIO NP dispersion and carried the particles through the subsequent drying assembly into the animal chamber. The animal chamber comprised of a 4-port double walled chamber. The dried aerosol stream entered the top inlet into a stirred chamber. The animals were placed into each port with their snout exposed to the aerosol stream in the stirred chamber. The exhaust tube was connected to the space between the two walls, thus ensuring that the aerosol stream passed by the mice nostrils before leaving the chamber. The mice were allowed to breathe in the aerosol normally for 30 minutes. Filter collections were made three times before the mice exposure and thrice after the end of the exposure to calculate the aerosol output. The aerosol stream was also passed through a 7-stage Intox cascade impactor before each exposure to determine the aerosol particle size distribution (mean median aerodynamic diameter and geometric standard deviation)[4]. The characteristics of the aerosol stream were consistent between the different experimental runs. The average aerosol output was 270 ± 70 µg/min. The mean median aerodynamic diameter was 1.1 ± 0.1 µm with a geometric standard deviation of 1.9 ± 0.1.

Distribution of SPIO nanoparticles following instillation and inhalation delivery

TPCP and SPCP were administered into the animals (n = 6 per time point per group) by the two routes of administration described above. The animals were euthanized at 1 hour, 1 day or 1 week after SPIO administration. The lungs, liver, spleen, kidney, heart, stomach and blood were collected to analyze the distribution of SPIO particles. Additionally 6 healthy mice were also euthanized and the organs collected to determine the basal level of iron in each organ. Lungs of healthy mice receiving inhaled or instilled SPIO particles were collected 1 hour after administration, formalin fixed, paraffin embedded and sectioned. Hematoxylin and eosin staining (H & E staining) and Prussian blue staining (for iron)[6] were performed to visualize the distribution profile of the particles after each method of administration. Additionally, lungs of treated animals were also sectioned and imaged after the last time point to visualize the distribution of SPIO particles.

Following instillation, SPIO particles were mostly observed in the lungs after 1 hour, although some particles were also present in the stomach probably due to the clearance of the particles from the trachea into the throat. There was no statistically significant difference between the levels of TPCP or SPCP in the different organs after 1 hour or 1 day after instillation. The concentration of both TPCP and SPCP in blood and other organs (except lung) decreased 1 week after instillation compared to 1 hour or 1 day time points (not shown). On the contrary, while the lung concentration of SPCP decreased over the period of 1 week, the level of

TPCP was fairly constant over the period. The final concentration of TPCP was significantly higher than SPCP (60 % more than SPCP) one-week post instillation **(Fig. 4)**. The final iron oxide content of the different organs after a week of instillation is also shown in **Fig. 4**.

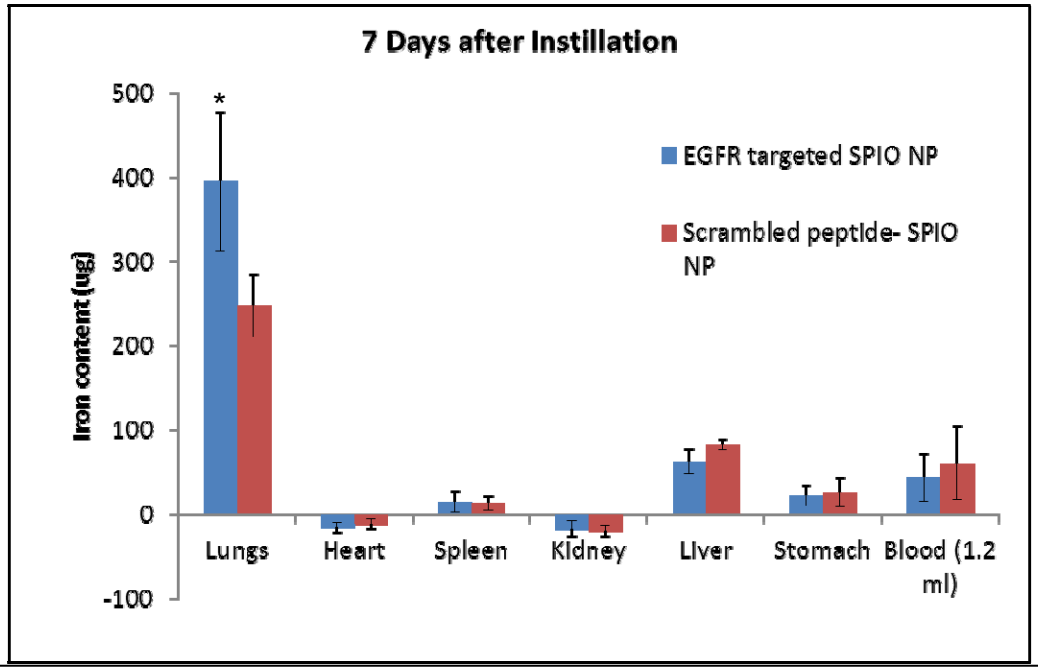

Fig 4: SPIO NP concentration of different organs 1 week after tracheal instillation. The lung concentration of EGFR-targeted SPIO NP was significantly higher ($p<0.05$) than that of the non-targeted scrambled-peptide conjugated SPIO NP.

Following aerosol exposure, the iron oxide concentration in the lungs at 1 hour **(Fig. 5)** was similar for TPCP and SPCP in tumor bearing lungs as well as for blank SPIO particles in healthy lungs, showing that the presence of tumor didn't affect the total inhalation intake of the particles. It also suggested that there is no role of targeting immediately after administration of the particles. However, after a week following inhalation, the lung concentration of SPCP decreased while the level of TPCP was almost constant. The significant higher concentration of TPCP demonstrates the effectiveness of EGFR targeting in improving particle retention in the lungs.

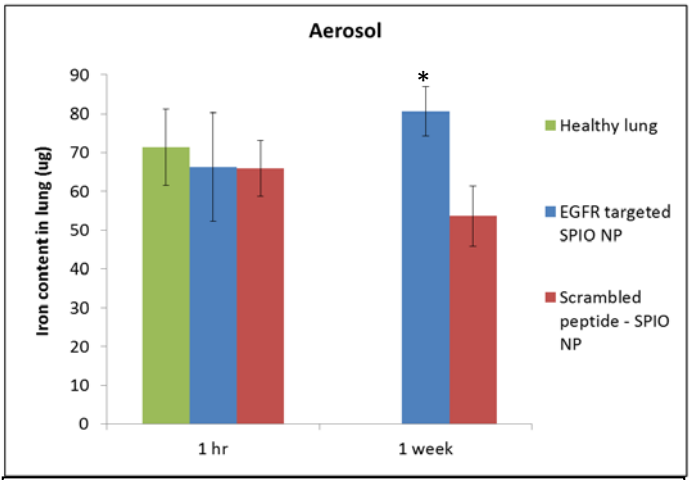

Fig 5: SPIO NP concentration in lung 1 hour and 1 week after inhalation of the particles. The lung concentration of EGFR-targeted SPIO NP was significantly higher ($p<0.05$) than that of the non-targeted scrambled-peptide conjugated SPIO NP.

While both the routes of administration showed a significant increase in lung concentration of the particles with EGFR targeting, the absolute amount of SPIO particles in the two cases were different. The lung iron content after instillation was 4.5 to 5-fold

higher than that after inhalation (400 vs 80 μg for EGFR-targeted SPIO nanoparticles, Fig. 4 and 5). This is due to the greater flexibility of the instillation in increasing the amount of SPIO delivered to the lung simply by changing the concentration of initial SPIO suspension instilled. However, aerosol delivery is a more acceptable route of administration in humans than instillation. Additionally, inhalation results in an even distribution of the SPIO through the lungs at the time of administration compared to instillation where the major amount of the instilled dose resides near the major airways with almost no particles reaching the periphery of the lungs.

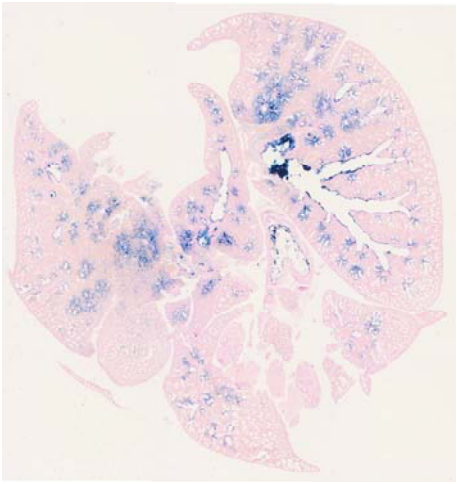

Fig 6. Prussian blue staining (for iron) of lung section 1 week after instillation showing the heterogeneous distribution of the SPIO NP in the lung.

The distribution profile of the particles in the healthy lung after instillation can be seen in **Fig. 6,** where the Prussian blue staining (for iron) is denser near the major airways. In contrast, inhalation results in a more homogenous distribution of the particles throughout the lung **(Fig. 7)**. One week after instillation in tumor bearing mice, a significantly higher amount of Prussian blue staining was observed in the TPCP-administered groups compared to SPCP-treated groups **(Fig. 8)**. Interestingly, the staining seemed to be localized into isolated cells for both the treatments. Similar differences in iron staining were seen one week post inhalation. In the TPCP, the staining was observed in and around tumor cells with almost complete absence of particles from the healthy parts of the lung. SPCP group didn't display much staining in either the tumor or the healthy regions of the lung (not shown).

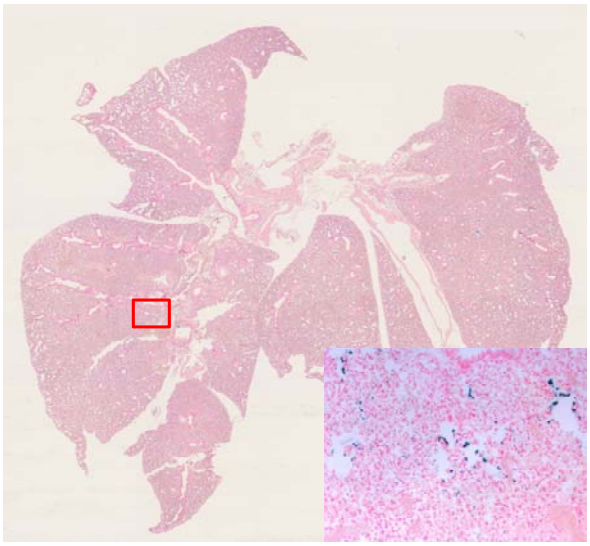

Fig 7. Prussian blue staining of lung section 1 week after inhalation with the high magnification inset showing a relatively homogeneous distribution of the SPIO NPs.

This study provides a basis for the selection of the administration route for determining the efficacy of targeted magnetic hyperthermia against lung cancer. Better lung distribution can be achieved using inhalation delivery while a higher particle concentration can be achieved using instillation.

Task 2: Study effect of inhalation dose on tumor concentration of SPIO nanoparticles

The previous study showed that irrespective of the route of administration, targeting enhances the lung/tumor concentration of the SPIO particles by 50 – 60% a week after delivery. The dose delivered via inhalation can be altered by increasing the initial concentration of the particles to be aerosolized and the duration of exposure to the aerosol. Since the SPIO NPs have a high density, increasing the SPIO concentration would result in a subsequent increase of the viscosity of the medium and decreased aerosol output[7,8]. Additionally, changing initial concentration of the particles resulted in an alteration of the mean median aerodynamic diameter of the particles, which can reduce the lung deposition fraction. In a preliminary study to measure aerosol output at different concentrations of SPIO particles, we observed that an initial particle concentration of 8 mg/ml resulted in the highest aerosol output and hence was used this does for the PK study.

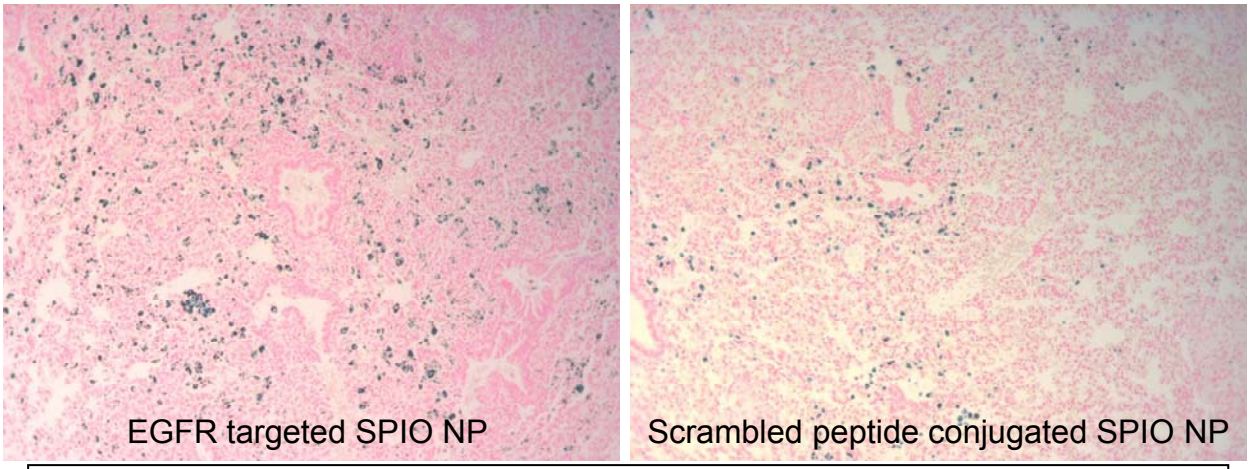

Fig 8. 100X magnification of the Prussian blue stained lung sections from animals that received instillation of either the EGFR-targeted SPIO NP or scrambled peptide conjugated SPIO NP.

The duration of exposure in the previous study was 30 minutes. While a higher duration of exposure could lead to a higher lung concentration, it is impractical to perform such long durations for experimental purposes. On the other hand, reduced exposure duration can be used to obtain a lower inhalation dose. Since we did not see any toxicity with the highest dose, we did not see a need to decrease the concentration of particles in the lungs.

It was relatively easier to increase the administered dose of particles following instillation. While an increased concentration of the particles can result in a higher quantity of SPIO delivered, such high concentrations can negatively affect the stability of the particles. Besides, increased concentration would further increase the viscosity of the solvent, further hindering the lung distribution of the particles. For these reasons, further modification of the inhalation dose was not attempted.

Specific Aim #3: Determine the in vivo anticancer efficacy of inhaled EGFR-targeted SPIO nanoparticles

We have started performing studies in Specific Aim 3 recently.

Task 1: Determine maximal tolerated dose of targeted SPIO nanoparticles

In the previous section, we discussed the difficulty in increasing the dose of SPIO NP into mice lungs. Hence the dose administered during the distribution study was the maximum dose that will be administered to the animals. One week after inhalation or instillation of the particles, the mice appeared healthy and did not show any abnormality in their behavior. The animals exhibited no obvious signs of distress (hunched posture, ruffled fur, difficulty in breathing) over the period of one week. Additionally the concentration of particles in the body after 7 days of inhalation or instillation was rather low **(Fig. 4 and 5),** except for the tumor bearing lungs. The iron concentrations were sub-lethal and seemed to decrease over time. Thus no severe toxicity is expected from the dose of SPIO NP administered to the animals via either of the two routes of administration.

KEY RESEARCH ACCOMPLISHMENTS

- Demonstrated effective cellular uptake and cell kill with EGFR-targeted SPIO particle-mediated magnetic hyperthermia.
- Demonstrated the effectiveness of EGFR targeting in enhancing the lung tumor concentration of SPIO particles in a mouse model.
- Started examining the toxicity and efficacy of targeted SPIO particle-mediated hyperthermia in a lung tumor model.

REPORTABLE OUTCOMES

- **Magnetic Hyperthermia for Lung Cancer**
  - Rho Chi Research Symposium 2011, Minneapolis, MN (poster)
  - Pharmaceutics Department Seminar 2012, Minneapolis, MN (oral)
- **Magnetic Hyperthermia as an Effective Approach to Kill Cancer Stem Cells**
  - AAPS 2011, Washington, DC (poster)
  - 7th Annual Minnesota Nanotechnology Workshop 2011, Minneapolis, MN (poster)
  - Rho Chi Research Symposium 2012, Minneapolis, MN (poster)
- **Acute necrosis and reactive oxygen species generation by magnetic hyperthermia leads to effective elimination of cancer stem cells**
  - 9th International Conference on the Scientific and Clinical Applications of Magnetic Carriers 2012, Minneapolis, MN (poster)
- **Iron oxide particle size determines the anticancer effectiveness of magnetic hyperthermia**
  - IPRIME 2012, Minnespolis, MN (oral)

CONCLUSION

Enhanced tumor cell uptake and *in vivo* mouse lung retention of SPIO NPs functionalized with EGFR targeting ligand was demonstrated compared to non-functionalized or scrambled peptide conjugated nanoparticles. No severe toxic manifestations were observed from the dose of SPIO NP administered to the animals. Future studies will examine the anti-tumor efficacy of the EGFR-targeted SPIO nanoparticles.

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APPENDICES

None

SUPPORTING DATA

All supporting data have been included as a part of the main body of this progress report.